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HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON*

Fourth Quarterly Report of Progress

on

Research Project Number 4E04-14-004
Order Number FDO-5013

April 1 - June 30, 1961

Conducted by

Milk and Food Research, SEC

for the

U. S. Army Chemical Corps Biological Laboratories
Fort Detrick, Maryland

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio
July 1961

*"In conducting the research reported herein, the investigator(s) adhered to 'Guide for Laboratory Animal Facilities and Care' established by the Committee on the Guide for Laboratory Animal Facilities and Care' of the Institute of Laboratory Animal Resources, NAS-NRC"

Unclassified Reports:

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HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

I. Introduction

The purposes for undertaking this project are (1) to determine the feasibility of joining paralytic shellfish poison (PSP) with other molecules to produce conjugates having immunogenic properties, (2) to develop a serological microassay procedure for determining low levels of PSP, and finally, (3) to investigate the feasibility of the immunization of humans against PSP.

The preceding quarterly reports have summarized results of our investigations on the preparation and serological properties of conjugates resulting from coupling "diazo" PSP to ovalbumin and bovine γ-globulin. Although considerable progress had been made in the development of techniques for synthesis and the serological studies, only during the period covered by this report has evidence been developed that the "diazo" derivative of PSP has haptenic properties. The conjugates used for these investigations are the same as those described in the Third Quarterly Report (Modification III). The chemistry section of this report deals with some of the auxiliary studies which have been carried out in the course of preparing various derivatives of PSP.

II. Experimental

Immunological studies related to determining the antigenicity of PSP-ovalbumin and PSP-bovine gamma II globulin.

Investigations during the period Jan.-Mar. 1961 revealed that sera obtained from rabbits immunized separately with PSP-globulin, PSP-ovalbumin, globulin, and ovalbumin displayed high antibody titers to the conjugated antigens but exceptionally poor titers to globulin or ovalbumin alone. The latter phenomenon was reasoned to be related to the denaturation of the native proteins by alkalinization during preparation. Cross-reactions were obtained between anti-PSP-globulin sera and the PSP-ovalbumin antigen on one occasion, but was not observed again in several later attempts. The cross reaction indicated the possibility of a hapten-like antigen having been produced by the conjugation procedure.

Additional work since March with the same antigen preparations and anti-sera has been directed chiefly toward demonstrating a hapten-inhibition-type reaction in which toxin (PSP) or diazo-toxin (D-PSP) specifically inhibits the reaction between the conjugated antigens and homologous anti-sera.

Due to the limited supply of toxin and relatively low concentrations available in solution, an inhibition test performed in the equivalence zone, as is done conventionally, appeared difficult to achieve. In order to note inhibition employing very small concentrations of inhibitor (PSP or diazo-PSP), the test necessarily would have to be run in the area of antigen excess. In such a situation, the relatively few antibody particles present are saturated by the inhibitor and not free to react with the homologous antigen (PSP-ovalbumin or PSP-globulin) upon subsequent addition to the system. On the other hand, performing an inhibition test with small concentrations of inhibitor versus relatively large concentrations of antibody results in combination of all the inhibitor to relatively little of the available antibody yielding free antibody to react with homologous antigen.

In order to avoid the latter situation, PSP-globulin was diluted in saline 1:256 and PSP-ovalbumin 1:2048. These concentrations represented the highest dilution of each which yielded maximum precipitate in the presence of a 1:5 dilution of homologous antiserum. In turn, the antisera to these antigens were diluted 1:2.5, 1:5, 1:10, 1:15, 1:20, and 1:40. Each of the dilutions of sera were reacted with the homologous antigen diluted as above and the highest dilution of antibody which consistently yielded demonstrable precipitate was recorded. By this process, it was possible to select for each serum that dilution which, when incubated with antigen, resulted in a reaction in the zone of antigen excess. Having established the serum dilutions necessary, inhibition tests were performed employing PSP or diazo-PSP as the inhibitor. To each of a series of precipitin tubes was added 0.2 ml. of the indicated serum dilution. To each tube was added 0.2 ml. of a doubling dilution of inhibitor prepared in distilled water. The tubes were incubated in a 37°C. water bath for 30 minutes. Following the initial incubation, 0.4 ml. of PSP-globulin (1:256) or PSP-ovalbumin (1:2048) was added to each tube and the tubes incubated for an additional 30 minutes at 37°C. After incubation, the tubes were placed in a 5°C. cold room and held overnight. Following incubation in the cold, the tubes were centrifuged at 2,000 r.p.m. and observed for the quantity of precipitate formed. Employing this procedure, it was possible to demonstrate the diazo-PSP inhibited the reaction between PSP-globulin-anti-PSP globulin and PSP-ovalbumin-anti-PSP-ovalbumin systems. Toxin alone (PSP) did not inhibit these reactions. The inability of PSP to inhibit the reaction between conjugated antigens and homologous antisera reveals the specificity of the antibody developed to be directed toward diazotized PSP and not PSP alone. A summarization of these data is presented in Table 1.

Because diazo-PSP is prepared and used in the inhibition test as a solution containing considerable excess nitrite ions (240 mMoles $NaNO_2$ and 41.3 mMoles HCl for each mMole of poison), inhibition tests were conducted employing an excess nitrite solution of comparable strength to that used to prepare the diazo-PSP. These tests revealed that the excess nitrite ion did not interfere with the reactions between the conjugated antigens and their homologous antisera. To further establish the specificity of the inhibition, diazo-PSP was introduced as an inhibitor into globulin-anti-globulin and ovalbumin-anti-ovalbumin systems to determine whether the highly reactive diazo-PSP molecule affects antibody globulin nonspecifically, giving rise to false-positive inhibition tests. These tests revealed that diazo-PSP had no effect on the reaction between these systems and provided additional evidence that diazo-PSP is active as an inhibitor by reacting with antibody directed against protein conjugated PSP. These data are presented in Tables 2 and 3.

To further demonstrate antibody directed toward PSP, a series of skin tests was performed with actively and passively immunized rabbits. Rabbits 36 and 38, actively immunized with PSP-globulin and PSP ovalbumin, respectively, were injected intradermally with 0.1 ml. quantities of the following antigens: PSP globulin, globulin, PSP ovalbumin, ovalbumin and saline. Reactions were recorded at various intervals throughout a 6-day period. These data are presented in Tables 4 and 5 and reveal cross-reactions of PSP globulin to anti-PSP ovalbumin and PSP ovalbumin to anti-PSP globulin occurred.

To establish that the antibody reacting in the inhibition tests, and in the above described skin reactions, was capable of being passively transferred, the following experiment was conducted. Normal (non-immunized) rabbits numbered 53, 54, and 55 were obtained. From rabbit 53, twenty-five ml. of blood was removed and replaced with an equal volume of a 50% suspension of blood cells in saline obtained from rabbit 36 which was previously immunized with PSP globulin. From rabbit 54, 15 ml. of blood was removed and replaced with an equal volume of plasma from rabbit 36. Rabbit 55 received no treatment. The rabbits were rested overnight and the following morning injected intradermally on the dorsal surface with 0.1 ml. volumes of the following antigens: PSP globulin, PSP ovalbumin, globulin, ovalbumin, excess nitrite solution, and saline. Skin reactions were recorded at various intervals over a 4-day period. Rabbit 53 (received formed elements of the blood) displayed no reaction to any antigen save excess nitrite solution. At the site of injection of the latter, a mild erythema developed within a few hours and disappeared within 48 hours. This was recorded as a non-specific reaction, due to irritation by the physiologically unacceptable nitrite solution. Rabbit 54 (received immune plasma) developed typical skin reactions at the sites of injection of PSP globulin, globulin and PSP ovalbumin. A nonspecific reaction to excess nitrite solution also developed and persisted for approximately 48 hours. Rabbit 55 (untreated control) developed the nonspecific reaction to excess nitrite solution. No reaction at the remaining sites of injection was observed. These data are summarized in Table 6 and indicate the antibody produced in response to PSP conjugated protein is of the circulating type and passively transferable.

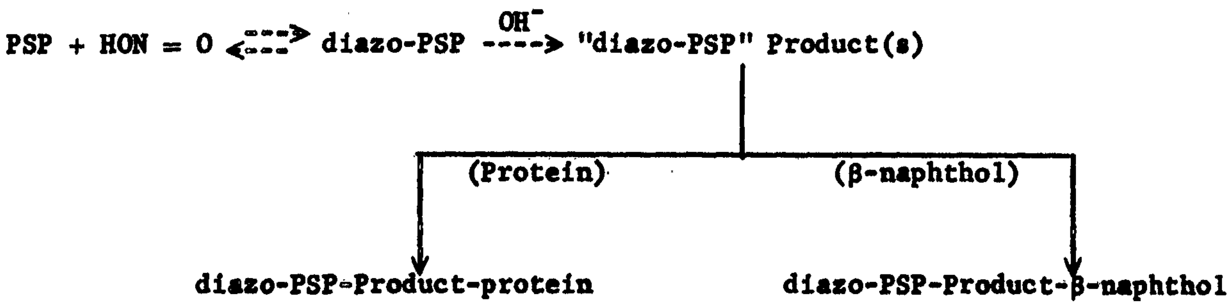
To determine the protective nature of the diazo PSP antibody, a series of 2 mice each were injected I.P. with saline, normal serum, anti-PSP globulin serum, and anti-PSP ovalbumin serum given as two 1.0 ml. injections 24 hours apart. Four hours following the second injection, the mice were challenged with 0.3 microgram of PSP contained in 0.1 ml. of aqueous solution. All the mice receiving sera died within 5 1/2 to 8 minutes after injection. Mice which received saline died within 4.0 to 4.3 minutes. Because mice receiving immune sera died as rapidly as mice receiving non-immune serum, no significance was attributed to the difference in death time observed for saline control mice versus serum protected mice. The time differential was assumed to be related to the rate at which the toxin was absorbed in the presence of residual concentrations of serum in the peritoneal cavity. In vitro neutralization tests were also performed with similarly negative results.

These data indicate that a method has been developed by which PSP may be conjugated to proteins to serve as an antigen. Antibody specificity is directed toward the diazo-toxin entity and little, if any, to toxin. The antibody produced may be transferred passively, but exhibits no protective properties.

Some Chemical Characteristics of PSP

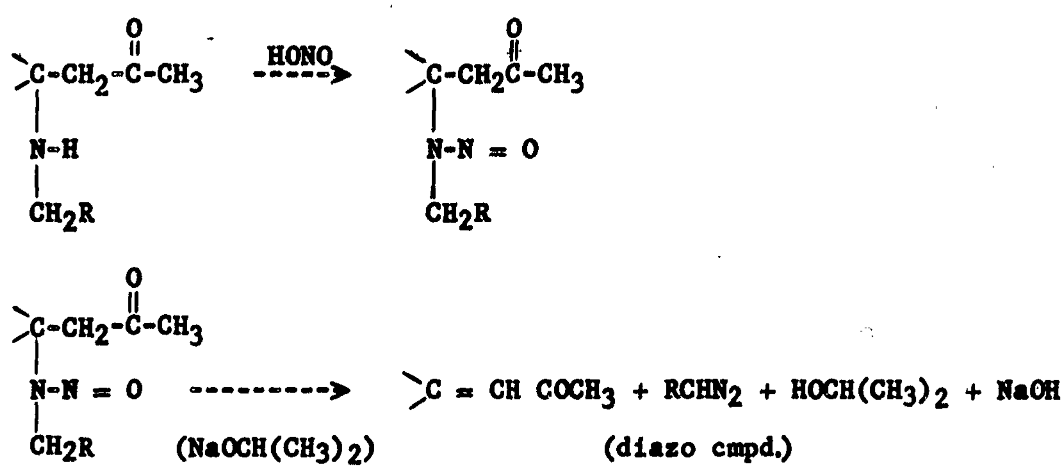
The chemical reactivity of PSP with various reagents has been rather carefully investigated. These reagents include nitrous acid, β-naphthol, aromatic diazonium salts, and 1,2-naphthoquinone-4-sulfonic acid (Na salt).

The most noteworthy of these, since it is involved in the coupling of PSP to proteins, is the reaction of PSP with nitrous acid resulting in the formation of a soluble yellow reaction product and accompanied by a 99% loss in toxicity. Toxicity studies, previously described in the Third Quarterly Progress Report (page 7), show that in such a solution a large part of the PSP toxicity can be recovered by removing the excess nitrous acid. These findings indicate that so long as PSP and nitrous acid are held in acid solution, they enter into a reversible reaction and suggest that PSP is not greatly altered by this treatment. However, since the coupling to proteins and β-naphthol occurs in alkaline solutions, it has been of more interest to determine, if possible, the extent of any change undergone by "diazo-PSP" under those conditions. Previous observations have shown that "diazo-PSP" undergoes a marked increase in color upon raising the pH of the solution. Once the pH of the solution has been raised above 5 in the absence of β-naphthol, it is no longer possible to visualize a reaction with β-naphthol. Similarly, attempts to increase the toxicity of "diazo-PSP" solutions, which have been made alkaline and immediately reacidified prior to the removal of excess nitrous acid, have been unsuccessful. The following scheme may represent a part of the changes involved.



Some of the possible reactions of aliphatic compounds with nitrous acid, which may be relevant, are illustrated in Table 7.

To draw any conclusions from such incomplete information as that in Table 7 would be a mistake. However, certain types of reactions can be immediately eliminated on the basis of color alone. Thus N-nitrosation and diazoacetate formation emerge as the most likely types of reactions. It should be noted that certain types of N-nitroso compounds called "nitrosamines" are converted to aliphatic diazo compounds under alkaline conditions. However, this usually results in a major change in chemical structure. The following may serve as an illustration.

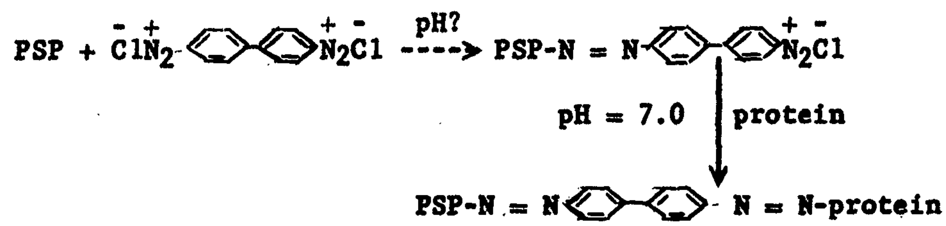


This is just the same reaction which occurs when the N-nitrosated derivative of methyl urea is subjected to alkaline conditions to form diazomethane.

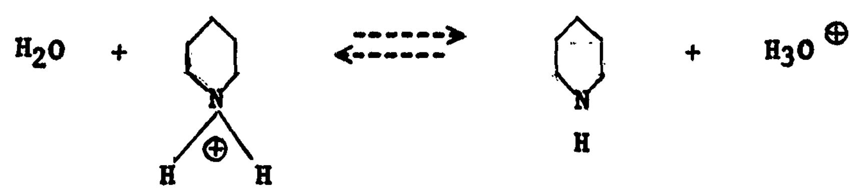
$$\mathrm{NH_2{-}\overset{\overset{O}{\|}}{C}{-}\underset{\underset{N\,=\,O}{|}}{N}{-}CH_3} \xrightarrow{\mathrm{OH^{\ominus}}} \underset{\text{(diazomethane)}}{\mathrm{CH_2N_2}} + {}^{\ominus}\mathrm{OCN} + \mathrm{H_2O}$$

Hence the possibility exists that the PSP molecule may be entirely cleaved under the alkaline conditions of coupling.

Purified PSP reacts with aromatic diazonium salts to form a product(s) having a yellow color. Since the reaction does not involve the evolution of nitrogen gas, it is likely that some chemical linkage between PSP and aromatic diazonium salts is established. The product(s) of reaction between PSP and diazotized sulfanilic acid absorbs light of 340 mμ wave length with a molar absorbancy index of 1300. There is little hope for a chemical micro-assay by this route, however, owing to the low absorbancy index and non-specific reactivity of aromatic diazonium salts. The primary interest in this reaction has been to see if PSP can be coupled to one site of a bifunctional diazonium salt and the remaining site to some protein. This would afford a linkage between PSP and protein which would involve a minimum alteration in PSP. Also such a process could be carried out rapidly, at low temperature and in nearly neutral solutions. The problem encountered has been the multiplicity of sites on PSP reactive toward diazonium salts. This coupling is pH dependent. For example, at pH 4.6 and pH 6.0 there appears to be no permanent bonding between PSP and aromatic diazonium salts. However, in one experiment at pH 7.0, benzene diazonium chloride appeared to couple to five sites on PSP. This experiment was performed with a somewhat impure sample of PSP, however, and should be repeated. Before such a system can be worked out, it is necessary to find the pH conditions at which only one site on PSP is reactive toward aromatic diazonium salts, so that the following reaction sequence can be carried out.



Such a system has been worked out with piperidine as a model compound. It is not a very satisfactory model, however, since an equilibrium is established at pH = 7.0 in which only about 35% of piperidine is availa-



ble in the free unprotonated form required for coupling to diazotized benzidine. Probably over a period of time the reaction would be driven to the right by the removal of free piperidine in the coupling, but time is an important factor in preserving the highly reactive diazonium salts.

Little work has been undertaken on the reaction of sodium 1,2-naphtho-quinone-4-sulfonate with PSP at pH 9.35 due to the insufficient quantities of PSP available. The product of this reaction is faintly violet and has U.V. absorption at 242 mμ with a molar absorbancy index of 15,400. Histamine, creatinine, and piperidine also react to form species absorbing in the 240 mμ regions; however, histamine is the only one of these known to be present in a sample of partially purified poison. Other substances known to be present are tertiary amines which do not interfere. It may be possible either to selectively remove histamine from a sample of partially purified poison or to determine the histamine separately and correct the U.V. data for PSP.

III. PROJECTED RESEARCH FOR FIRST QUARTER, FY 1962

Projected research for the first quarter, FY 1962 will be directed toward the following objectives:

1. Investigate alternate methods for coupling paralytic shellfish poison to proteins under conditions which will allow for even less change in the configuration of the paralytic shellfish poison molecule.

2. Develop more rigorous evidence concerning the nature of the diazotized paralytic shellfish poison-protein conjugates through the use of physical techniques such as electrophoresis and ultracentrifugation.

3. Undertake additional immunological and serological studies on the "diazo" PSP-Protein conjugate described in the Third Quarterly Report of Progress to gain additional information concerning alternate serological methods for demonstrating the antigen-antibody reaction.

4. Make preliminary studies on the usefulness of the haptenic properties of "diazo" PSP as a microassay tool.

IV. SUMMARY

Rabbit antisera produced to protein conjugated PSP contain precipitating antibodies of high titer. Inhibition tests, employing PSP and diazotized PSP, reveal that the reaction of PSP-globulin and PSP-ovalbumin to their homologous antisera is specifically inhibited by diazotized PSP. The antibodies produced to the conjugated proteins are passively transferrable as indicated by skin tests in normal rabbits. *In vivo* and *in vitro* tests to neutralize toxin by combination with anti-protein conjugated PSP sera yielded negative results. Some of the possible chemical reactions of PSP are discussed with special emphasis on the reaction with nitrous acid.

Table 1

Inhibition of reaction between PSP-globulin and PSP-ovalbumin antigens and anti-PSP-globulin and anti-PSP-ovalbumin sera by diazotized PSP

No. of Serum	Dilutions of anti-sera 0.2 ml./tube	Antigen dilution 0.4 ml./tube	Saline 0.2 ml. per tube	Micrograms of PSP per tube 0.2 ml. per tube						Micrograms of diazotized toxin per tube equivalent to micrograms of toxin 0.2 ml. per tube.					
				90	45	22.5	11.3	5.6	2.8	90	45	22.5	11.3	5.6	2.8
36	Anti-PSP-globulin 1:15	PSP-globulin 1:256	+	+	+	+	+	+	+	-	-	±	+	+	+
37	Anti-PSP-globulin 1:10	PSP-globulin 1:256	2+	2+	2+	2+	2+	2+	2+	-	-	2+	2+	2+	2+
41	Anti-PSP-globulin 1:10	PSP-globulin 1:256	3+	3+	3+	3+	3+	3+	3+	-	+	2+	2+	3+	3+
38	Anti-PSP-ovalbumin 1:2.5	PSP-ovalbumin 1:2048	3+	3+	3+	3+	3+	3+	3+	+	2+	3+	3+	3+	3+
39	Anti-PSP-ovalbumin 1:5	PSP-ovalbumin 1:2048	+	+	+	+	+	+	+	-	-	+	+	+	+
40	Anti-PSP-ovalbumin 1:2.5	PSP-ovalbumin 1:2048	2+	2+	2+	2+	2+	2+	2+	+	+	+	2+	2+	2+

Table 2

Non-inhibitory effect of excess nitrite solution upon reaction of anti-PSP-globulin serum with PSP-globulin antigen

Serum number	Antibody	Antigen	Saline	Dilutions of excess nitrite solution (Concentrations equivalent to those existing in tube dilution series of diazo PSP)					
				1:2	1:4	1:8	1:16	1:32	1:64
37	Anti-PSP globulin 1:5	PSP-globulin 1:256	4+	4+	4+	4+	4+	4+	4+
37	Anti-PSP globulin 1:5		-						
37	Anti-PSP globulin 1:5	Saline		-					
		PSP-globulin 1:256	-						

Table 3

Non-inhibitory effect of diazo PSP on globulin-anti globulin and ovalbumin-anti ovalbumin systems

Tube number	Serum number	Antibody	Antigen	Saline	Diazo-PSP 90 μg.
1	48	Anti-globulin 1:5	globulin 1:32		4+
2	48	Anti-globulin 1:5	globulin 1:32	4+	
3	48	Anti-globulin 1:5		-	
4			globulin 1:32	-	
5	43	Anti-ovalbumin 1:5	ovalbumin 1:1000		4+
6	43	Anti-ovalbumin 1:5	ovalbumin 1:1000	4+	
7	43	Anti-ovalbumin		-	
8			ovalbumin 1:1000	-	

Table 4

Skin reaction of rabbit (36) immunized with PSP-globulin and having homologous serum titer in excess of 1:20,000 (0.1 ml. of each antigen injected I.D., dorsal surface)

Hours after injection	Reactions at site of injection of:				
	PSP-globulin	globulin	PSP-ovalbumin	ovalbumin	saline
1	slight swelling	slight swelling	normal	normal	normal
2	erythema 1.0 cm. area slight swelling	erythema 1.5 cm. area swelling	small papule	normal	normal
3	erythema 1.5 cm. area slight swelling	erythema 1.5 cm. area swelling	small papule	normal	normal
4.5	erythema 2.0 cm. area slight swelling	erythema 2.0 cm. area	normal	normal	normal
22	severe erythema 2.5 cm. area no swelling	severe erythema 2.5 cm. area no swelling	slight erythema 1.0 cm. no swelling	normal	normal
25	severe erythema 2.5 cm. area no swelling	severe erythema 2.5 cm. area no swelling	slight erythema 1.0 cm. no swelling	normal	normal
29	erythema fading 1.5 cm. area no swelling	erythema fading 2.5 cm. area no swelling	erythema very faint 1.0 cm. area no swelling	normal	normal
46	slight erythema 1.5 cm. area	slight erythema 2.5 cm. area no swelling	normal	normal	normal
70	erythema very faint 1.5 cm. area no swelling	erythema very faint 2.5 cm. area no swelling	normal	normal	normal
144	normal	normal	normal	normal	normal

Table 5

Skin reaction of rabbit (38) immunized with PSP-ovalbumin and having homologous serum titers in excess of 1:20,000 (0.1 ml. of each antigen injected I.D., dorsal surface)

Hours after injection	Reactions at site of injections of:				
	PSP-globulin	globulin	PSP-ovalbumin	ovalbumin	saline
1	slight swelling	normal	slight erythema slight swelling	slight erythema slight swelling	normal
2	slight erythema 0.75 cm. area swelling	slight swelling	erythema 0.75 cm. area swelling	erythema 1.25 cm. area swelling	normal
3	erythema 1.0 cm. area slight swelling	normal	erythema 1.0 cm. area swelling	erythema 1.0 cm. area swelling	normal
4.5	erythema 1.0 cm. area no swelling	normal	dark erythema 1.5 cm. area. Swelling	dark erythema 1.5 cm. area swelling	normal
22	erythema fading 1.0 cm. area	normal	severe erythema 1.25 cm. area. No swelling	severe erythema 1.5 cm. area no swelling	normal
25	erythema faint 1.0 cm. area	normal	severe erythema 1.25 cm. area no swelling	severe erythema 1.5 cm. area no swelling	normal
29	erythema very faint 1.0 cm. area. no swelling	normal	severe erythema 1.5 cm. area no swelling	severe erythema 1.5 cm. area no swelling	normal
46	erythema barely visible and diffuse no swelling	normal	severe erythema 1.5 cm. area no swelling	severe erythema 1.5 cm. area no swelling	normal
70	normal	normal	erythema fading 1.5 cm. area no swelling	erythema fading 1.5 cm. area no swelling	normal
144	normal	normal	normal	normal	normal

Table 6.

Skin reactions of rabbits passively immunized with blood cells and plasma from a PSP-globulin immunized rabbit

Treatment	Hrs. after injection	Reactions at site of injection of: PSP globulin	Globulin	PSP ovalbumin	Oval-bumin	Excess nitrite solution	Saline
Rabbit 53 passively immunized with "immune" blood cells	6	normal	normal	normal	normal	normal	normal
	12	normal	normal	normal	normal	1.0 cm. erythema	normal
	24	normal	normal	normal	normal	1.0 cm. erythema	normal
	48	normal	normal	normal	normal	1.0 cm. erythema	normal
	72	normal	normal	normal	normal	normal	normal
	96	normal	normal	normal	normal	normal	normal
Rabbit 54 passively immunized with immune plasma	6	1.0 cm. erythema	1.0 cm. erythema	normal	normal	normal	
	12	1.5 cm. erythema	1.0 cm. erythema	1.0 cm. erythema	normal	1.0 cm. erythema	
	24	2.0 cm. erythema	2.0 cm. erythema	1.0 cm. erythema	normal	1.0 cm. erythema	
	48	2.5 cm. erythema	2.0 cm. erythema	1.5 cm. erythema	normal	1.0 cm. erythema	
	72	2.5 cm. erythema	2.5 cm. erythema	2.0 cm. erythema	normal	normal	
	96	2.0 cm. erythema	2.0 cm. erythema	2.0 cm. erythema	normal	normal	
Rabbit 55 untreated control	6	normal	normal	normal	normal	normal	normal
	12	normal	normal	normal	normal	1.0 cm. erythema	normal
	24	normal	normal	normal	normal	1.5 cm. erythema	normal
	48	normal	normal	normal	normal	1.0 cm. erythema	normal
	72	normal	normal	normal	normal	normal	normal
	96	normal	normal	normal	normal	normal	normal

Table 7.

Reactions of aliphatic compounds with nitrous acid

Reaction	Equation	Product Color
C-nitrosation	$-\overset{O}{\overset{\|}{C}}-\overset{\mid}{C}H- \xrightarrow{HONO} -\overset{O}{\overset{\|}{C}}-\underset{N=O}{\underset{\mid}{\overset{\mid}{C}}}- + H_2O$	Blue
	$-\overset{O}{\overset{\|}{C}}-CH_2 \xrightarrow{HONO} -\overset{O}{\overset{\|}{C}}-\underset{NOH}{\underset{\|}{C}}- + H_2O$	Colorless
N-nitrosation	$R-NH-R \overset{HONO}{\rightleftharpoons} -\underset{N=O}{\underset{\mid}{N}}- + H_2O$	Yellow
	$-\overset{O}{\overset{\|}{C}}-NH-R \overset{HONO}{\rightleftharpoons} -\overset{O}{\overset{\|}{C}}-\underset{N=O}{\underset{\mid}{N}}-R + H_2O$	Yellow
Nitrogen evolution	$RNH_2 \xrightarrow{HONO} R-OH+N_2\uparrow + H_2O$	Colorless
	$-\overset{O}{\overset{\|}{C}}-NH_2 \xrightarrow{HONO} -\overset{O}{\overset{\|}{C}}-OH + N_2\uparrow + H_2O$	Colorless
Aliphatic nitro compounds	$R_2-CH-NO_2 \xrightarrow{HONO} R_2 - \underset{N=O}{\underset{\mid}{C}} - NO_2$	Blue
	$R-CH_2-NO_2 \xrightarrow{HONO} R - \underset{N=O}{\underset{\mid}{CH}} - NO_2$	Red
Curtius re-arrangement	$R-\overset{O}{\overset{\|}{C}}-NH-NH_2 \xrightarrow{HONO} R-\overset{O}{\overset{\|}{C}}-N_3 + 2H_2O$	------
	$R-\overset{O}{\overset{\|}{C}}-N_3 \xrightarrow[\text{inert solvent}]{\text{heat}} R-N=C=O + N_2$	------
Diazo acetate formation	$RO-\overset{O}{\overset{\|}{C}}-CH_2NH_2 \xrightarrow{HONO} RO-\overset{O}{\overset{\|}{C}}-CHN_2 + 2H_2O$	Yellow